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Combining field epidemiological information and genetic diversity to understand *Phytophthora megakarya* dispersion in young cocoa plantations in Cameroon

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Phytophthora megakarya is the most virulent *Phytophthora* species reported on cacao (*Theobroma cacao*) in Africa. Previous studies have shown that it disperses mainly through rain splash from soil to pod where infection occurs. However, this mechanism takes place in already infected cacao plantations. How *P. megakarya* arrives in disease free plantations and what determines subsequent successful establishment are largely unknown. Disease monitoring with molecular tools can help to better understand dispersal mechanisms. The objective of this work is to identify the introduction pathways of *P. megakarya* in young cocoa plantations that could help predict and prevent further spread.

This study was carried out in Central-Cameroon on four cacao plantations, located in two distinct agro-ecological regions and established in 2006 on lands free of primary inoculum. These plantations were monitored on a weekly basis, from 2009 to 2016, for the presence of *P. megakarya*. As soon as first infections occurred, we started to collect *P. megakarya* in the field and from the surrounding environment. A total of 182 *P. megakarya* strains were isolated and genotyped using 14 polymorphic SSR markers.

Results indicate that disease incidence was relatively low from 2009 to 2016 and restricted to areas most conducive for disease development. The sampled *P. megakarya* populations showed limited genetic diversity. Thirty Multilocus Genotypes were obtained for all habitats but just one was constant over the years. Based on the spatial disease pattern observed in field and the occurrence of MLGs, it appears that the single constant MLG is the founder genotype which could be the main responsible for disease spread. The number of genotypes shared between the studied plantation and its surrounding environment suggests that inoculum originates primarily from neighboring cocoa plantations. Run-off water seems to be an important dispersal mechanism. The implications of these findings for *P. megakarya* control are discussed.

Keywords: *Phytophthora megakarya*, SSR markers, cacao, dispersal mechanisms

INTRODUCTION

Phytophthora megakarya, causing cacao black pod disease in West and Central Africa is the most virulent of the *Phytophthora* species reported on cacao (*Theobroma cacao*) (Cilas and Despereaux, 2004, Ali et al., 2016). In Cameroon, *P. megakarya* is a major production constraint and losses can easily reach 80-90% when no control measures are taken (Nyassé, 1997, Ndoumbè Nkeng, 2002). Similarly to many other *Phytophthora* spp, primary inoculum that initiates epidemics can be dispersed by multiple means e.g. insects, especially tent building ants, humans and water. However in the case of *P. megakarya* rain splash is the main dispersal mechanism (Ristaino and Gumpertz, 2000). In already well infected cacao plots, Gregory *et al*. (1984) determined that rain splash from soil or contact with infected pods was responsible for up to 71% of pod infections. Interestingly though, very little attention has been on the role of primary infections in *P. megakarya* epidemics. Though the soil was pointed as primary inoculum reservoir, from which initial infections derived directly or through ant tents harbouring pathogen propagules, other potential sources of primary inoculum are poorly studied (Griffin *et al.*, 1981).

Genetic studies of *P. megakarya* populations in West and Central Africa with isozyme and molecular markers (RAPD and SSR) show two strongly differentiated groups, one for Central and one for West-Africa. An intermediate group has been found which potentially could be the founder population from which both other groups have emerged. This intermediate group is located in the Cameroon-Nigeria border area and it has been suggested that this region could be the centre of origin of *P. megakarya* (Nyassé *et al*., 1999; Mfegue, 2012). *Phytophthora megakarya* is an invasive pathogen. It spread from central to West Africa where it is still in an emergent phase. *Phytophthora megakarya* seems to have displaced *P. palmivora* completely in Cameroon and Nigeria (Nyassé *et al*., 1999; Akrofi, 2015). Recent studies (Ali *et al*., 2016, 2017) provide insight into why *P. megakarya* is more virulent than *P. palmivora*. *P. megakarya* produces more appressoria than *P. palmivora* which consequently facilitates the infection process. It also seems to have greater diversity in virulence-related genes. All of which explain why *P. megakarya* continues to slowly spread in Ghana and Ivory Coast (Akrofi, 2015). In short, *P. megakarya* is a serious threat to the cacao economies of countries in Central and West Africa, and there is a need to develop sustainable management strategies.

Development of such management strategies is much more efficient when having among others, a thorough understanding of pathogen dispersal mechanisms. Several studies have demonstrated that the integration of population genetics and plant disease epidemiology can deliver solid information about dispersal mechanisms and enable the determination of sources of (primary) inoculum, and e.g. host specialization (Milgroom and Fry, 1997; Zwankhuizen *et al*., 1998; Milgroom, 2001). Since few studies have actually studied the dispersal mechanisms of *P. megakarya,* the relative importance of these mechanisms is not yet well identified. Therefore, the objective of this work was to identify introduction pathways of *P. megakarya* in young cocoa plantations through disease monitoring with SSR markers that could help predict and prevent further spread.

Material and methods

Study site

This study was carried out in the centre Region of Cameroon on four cacao plantations, located in two distinct agro-ecological zones. Plantations were established in 2006 in the villages of Bakoa, and Kedia (in the forest-savannah transitional zone, plots 1 and 2 in Bakoa & plot 3 in Kédia) and Ngat (in the forest zone, plot 4) on lands free of primary inoculum. The forest-savannah transitional zone is characterised by an intense dry season, a mean temperature range of 20 to 30 °C and rain fall of about 1100 mm yr^{-1}. Vegetation is primarily made up of gallery forest and grassland. The forest zone is more humid with a mean temperature range between 20 and 25 °C and rain fall of about 1600 mm yr^{-1}. Cocoa plantations were established in 2006 on areas varying from 3000 to 5000 m^2. Cocoa trees were either intercropped with *Elaies guineensis* (plot 1 and 3)*, Cocos nucifera* (Plot 2), or a mixture of fruit trees (*Citrus* spp*, Persea americana and Dacryodes edulis,* (plot 4). Spacing between cocoa trees was 3 x 3 m. The surrounding environment up to 100 meters away of these plantations was also characterised. Three of the plantations were close to already existing cacao plantations in which *P. megakarya* was already present. Plot 3 was also bordered by a river. All plantations had a slight but clear slope. For the remainder, the article will solely focus on data obtained from plot 3 located in Kedia.

Disease monitoring and isolate collection

The cacao plantations were monitored on a weekly basis, from 2009 to 2016, for the presence of *P. megakarya* pod infections. As soon as first infections occurred (end of 2009), *P. megakarya* was regularly collected from infected plots as well as from the surrounding environment. Soil samples were collected

twice a year (corresponding to the latent and fructification periods) in each plot. *P. megakarya* was isolated from soil samples using a cacao pod baiting technique previously used by Mfegue (2012). Between 2014 and 2015, *P. megakarya* was also sampled from the river bordering the plantation in Kedia and from run-off water. This way, a total of 182 *P. megakarya* isolates were obtained for plot 3 for genetic characterization (Table 1)

Table 1: Number of genotyped isolates and the number of MLG obtained in Kedia site

Population	Isolation habitat	Number of isolates	Number of MLG
Experimental plot	pod	91	17
	soil	28	7
Surrounding environment	pod	52	9
	water	11	9
Total		182	30

DNA extraction and genotyping

For DNA extraction, mycelia were picked off from a seven day old culture grown on standard V8 media and transferred to tubes containing 60 mL liquid V8 + Potato Dextrose Broth (PDB), and grown at 25°C for 7 seven days. DNA was extracted using a standard phenol-chloroform protocol and extracts were stored at −20°C. All 182 isolates were genotyped using 14 polymorphic SSR markers for *P. megakarya.* Eleven markers were previously developed by Mfegue *et al.* (2012) and three others in this study (unpublished results). Amplifications were performed using 10 µL reaction mixture consisting of 5 µL 1X QIAGEN Multiplex Master Mix, 1 µL 0.5X Q-Solution, 1 µL of 10X multiplex primers mix (0.2µM) and 3 µL DNA (20 ng μL^{-1}). PCR was performed in a thermocycler (PTC200, MJ Research) under the following conditions: the first one consist of initial denaturation at 94 °C for 15 min followed by 10 cycles of 94 °C for 30 s, 60 °C (slowdown 0.5 C per cycle) for 90s and elongation phase of 72 °C during 75s. The second one follows the previous directly with 30 cycles of 94 °C for 30s 55 C of 90s and final of 72 °C for 75s. The PCR products were diluted and mixed with a standard size LIZ500 (Applied Biosystems) and formamide then electrophoresis was performed using a capillary sequencer (Applied Biosystems). Allele size was calibrated using the reference strain NS269 used in previous studies (Nyassé, 1999; Mfegue, 2012) and a matrix of genotypes was obtained in GeneMapper and genetic diversity was determined by using GenAlex software.

Results

Disease monitoring

In Kedia, first black pod rot infections were observed in 2011. A total of 13 cacao trees harbored at least one infected pod that year. Due to logistical problems data from 2012 is incomplete. In 2013, 27 infected cacao trees were counted. In 2014, until the 04th of November, only 13 infected cacao trees were noted, while one week later 67 infected trees were counted. This is explained by a flooding event that occurred at this time. Finally, this lead to a total of 137 infected cacao trees at the end of 2014. Surprisingly this number was not maintained or increased in 2015 but reduced to 15, increasing to 23 in 2016. Apart from 2014, when black pod disease incidence reached 46% (137 infected trees out of 298) disease incidence was relatively low and stable, varying between 5-9% for the period under observation. It is also noteworthy to mention that in this particular plot, the trees closest to the river, especially in the eastern corner of the plot, were more often affected by the disease (Fig 1).

Until 2012, all soil samples collected in the experimental plot tested negative for the presence of *P. megakarya*. In 2013, nine samples tested positive with five of these collected under infected cacao trees. Five soil samples tested positive in 2014, 10 in 2015 and 18 in 2016. A total of 25 isolates were collected from run-off water and the river with 16 in 2014 and nine in 2015. Although some of the collected isolates were lost prior to genotyping, a total of 119 isolates from the experimental plot were genotyped with 91 from infected pods and 28 from soil samples. Isolates from water (11) as well as isolates from the surrounding environment (52), principally from infected cacao pods, were also genotyped (Table 1).

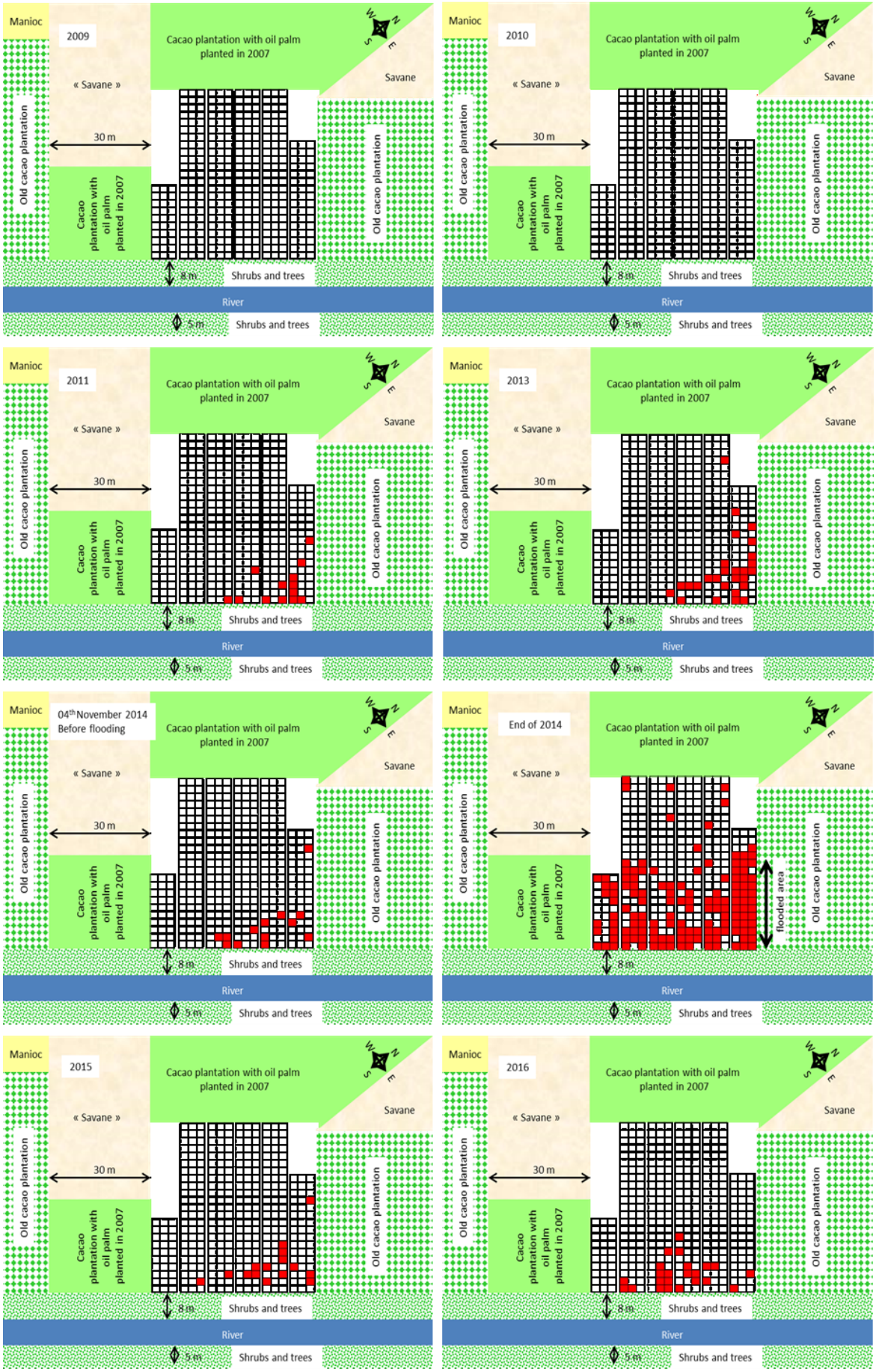

Figure 1: Spatial distribution of *Phytophthora megakarya* infections (2009-2016) in a cacao plantation in Kédia, established in 2006

Genetic characterization

A total of 43 alleles were detected over the 14 microsatellite loci with 2 to 8 alleles per locus. Because of insufficient number of isolates for each year, populations were defined according to the isolation line. The mean number of alleles per population varies from 2 to 3. The diversity index (Shannon's Index) was 0.43 and the fixation index value was -0,194 (table1). The 182 isolates of *P. megakarya* split into 30 Multilocus Genotypes (MLGs). The most representative (65% of all isolates) is MLG 11. From 2011 corresponding to the beginning of first *P megakarya* infection to 2016, several MLGs were observed in plot 3 with 17 from pods and seven from soil as well as from surrounding environment (9) and water (9). The occurrence of MLGs was not constant and the number of MLGs fluctuated throughout the years. The highest number (25) was obtained in 2014 while the lowest number (2) was observed in 2016. In all habitats, there were some unique MLGs while others were shared between plot and soil or plot and neighboring cacao plantations and/or river. Only MLG 11 was constant and shared between all habitats.

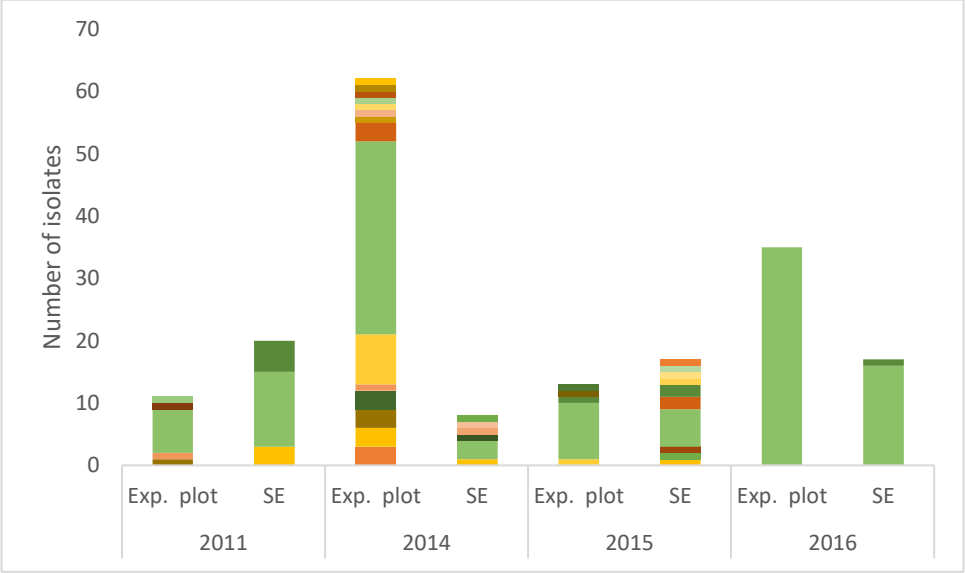

Figure 2: Distribution of *Phytophthora megakarya* MLG in experimental plot (Exp. Plot) and surrounding environment (SE) in time

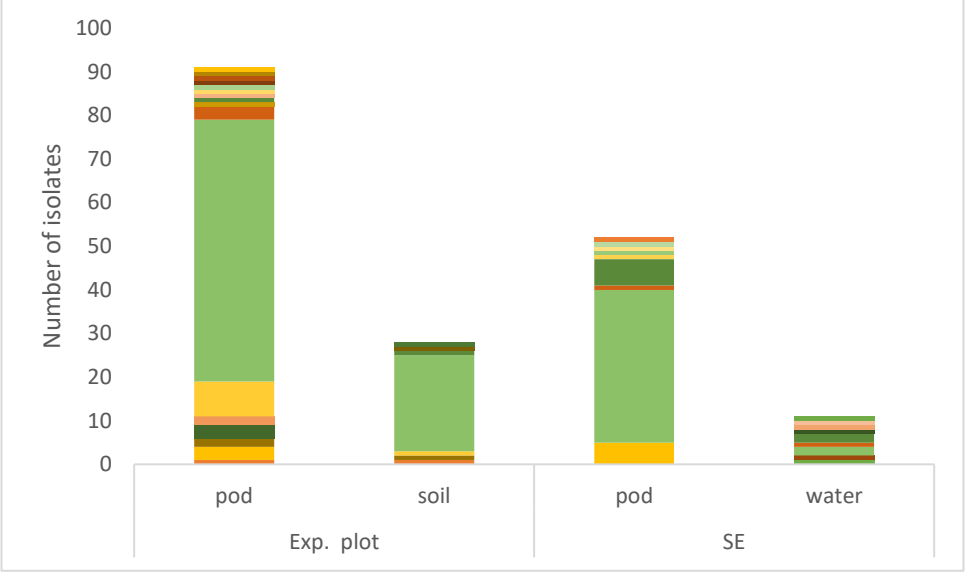

Figure 3: Distribution of *Phytophthora megakarya* MLGs obtained from pods, soil and water from the experimental plot (Exp. Plot) and the surrounding environment (SE)

Discussion

Map representation of the experimental plot with its surrounding environment allows visualization of the distribution of infected cacao trees over time. From 2011 to 2016 (except 2014), disease incidence was relatively low and restricted to the lower end of the slope which is also closest to the river.

Given that this plot was established in 2006 on land free of *P. megakarya* inoculum (until 2011, soil samples tested negative), the disease initiation hypothesis from soil was eliminated. Thus it appears that primary inoculum originated from neighboring cacao plantations. The observed pod rot pattern in 2011 towards the South-East as well as the genetic results of 2011 seem to support this idea, pinpointing to the old cacao plantation as the probable source of the first infections.

Although *P. megakarya* as well as many other *Phytophthora* species can spread through several distinct mechanisms, rain splash seems to be the main dispersal mechanism for *P. megakarya* (Gregory *et al*. 1984; Ristaino and Gumpertz, 2000). Soil samples that tested positive for presence of *P. megakarya* had 30% of MLG in common with pod samples, despite the relative low number of isolates obtained from soil. This flow between pod and soil was previously showed by Mfegue *et al* (2012). It could be explained by the fact that rain splash disperses inoculum not only from infected pods to healthy ones but also take it towards the soil and the movement could also go from soil to pod. This is what is generally assumed to happen trough rain splash. Yet, several MLGs were unique to pod (12), soil (2) and water (6) and indicate multiple introductions which could occur through other pathways.

The MLG 11 was present constantly in the field from 2011 to 2015 and in 2016 it is basically the only MLG, indicating that it is the founder and the main responsible of disease spread. This could be due to its fitness, which is its capacity to compete with others and to adapt to microenvironmental fluctuations.

For infection sources, Griffin *et al*. (1981) show that apart from soil, primary inoculum comes from ant tents (build with soil particles) and "no obvious sources". These "no obvious sources" probably encompass other dispersal mechanisms which have been cited as possible ways for inoculum dispersion but have not yet been studied. In the case of *P. palmivora* for example, flying insects might also be involved (Konam and Guest 2004), yet this remains to be confirmed for *P. megakarya*. The number of MLGs obtained from water indicated that run-off water is a potential habitat of *P. megakarya* inoculum. Such findings were found for other *Phytophthora* species (Themann *et al.,* 2002; Husson *et al*., 2006; Hüberli *et al.,* 2013). Based on this study and for this particular plot, it seems that run-off water and especially the river can disperse *P. megakarya* propagules. In conclusion, installing cacao plantation away from flood prone areas as well as by maintaining sufficient distance from already infected cacao plantations can be useful measures to prevent or delay the arrival of *P. megakarya*.

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